Behavioural and physiological responses to increased foraging effort in male mice

Vaanholt, Lobke M.; De Jong, Berber; Garland, Theodore; Daan, Serge; Visser, G. Henk

Published in:
Journal of Experimental Biology

DOI:
10.1242/jeb.001974

Publication date:
2007

The Journal of Experimental Biology 210, 2013-2024
Published by The Company of Biologists 2007
doi:10.1242/jeb.001974

Behavioural and physiological responses to increased foraging effort in male mice

Lobke M. Vaanholt[1,*], Berber De Jong[1], Theodore Garland, Jr[2], Serge Daan[1] and G. Henk Visser[1,3]

[1]*University of Groningen, Department of Behavioural Biology, Kerklaan 30, 9751 NN Haren, The Netherlands,* [2]*University of California, Riverside, Department of Biology, Riverside, CA 92521, USA and* [3]*University of Groningen, Centre for Isotope Research, Nijenborgh 4, 9747 AG Groningen, The Netherlands*

*Author for correspondence (e-mail: l.m.vaanholt@rug.nl)

Accepted 13 March 2007

Summary

Free-living animals must forage for food and hence may face energetic constraints imposed by their natural environmental conditions (e.g. ambient temperature, food availability). Simulating the variation in such constraints, we have experimentally manipulated the rate of work (wheel running) mice must do to obtain their food, and studied the ensuing behavioural and physiological responses. This was done with a line of mice selectively bred for high spontaneous wheel running and a randomly bred control line that vary in the amount of baseline wheel-running activity. We first determined the maximum workload for each individual. The maximum workload animals could engage in was around 23 km d^{-1} in both control and activity-selected mice, and was not associated with baseline wheel-running activity. We then kept mice at 90% of their individual maximum and measured several physiological and behavioural traits. At this high workload, mice increased wheel-running activity from an average of 10 to 20 km d^{-1}, and decreased food intake and body mass by approximately 20%. Mass-specific resting metabolic rate strongly decreased from 1.43 to 0.98 kJ g^{-1} d^{-1}, whereas daily energy expenditure slightly increased from 2.09 to 2.25 kJ g^{-1} d^{-1}. Costs of running decreased from 2.3 to 1.6 kJ km^{-1} between baseline and workload conditions. At high workloads, animals were in a negative energy balance, resulting in a sharp reduction in fat mass as well as a slight decrease in dry lean mass. In addition, corticosterone levels increased, and body temperature was extremely low in some animals at high workloads. When challenged to work for food, mice thus show significant physiological and behavioural adjustments.

Key words: mouse, daily energy expenditure, doubly labeled water technique, energy balance, resting metabolic rate.

Introduction

Free-living animals need to forage for food and they may face energetic constraints related to their natural environmental conditions, such as low ambient temperature and limited food availability. The main energetic costs for an endothermic and homeothermic animal with a large surface-to-volume ratio, such as a mouse, are of a thermoregulatory nature [rather than those related to costs of locomotion (Carbone, 2005; Garland, 1983; Goszczynski, 1986)]. Mice further need energy for maintenance of the body and for foraging activity. Excess energy can be used for non-essential physical activity, stored as fat or invested in growth and/or reproduction. When food is scarce, mice must invest more time (and energy) in foraging, and they may face constraints on the energy available for behaviour and maintenance functions other than foraging. They then need a physiological strategy to reallocate their limited energy. Fat reserves may provide energy for a short time (Bronson, 1987; Day and Bartness, 2001), but when food availability is low for extended periods animals must reallocate energy to systems that need it most from functions that are less crucial for survival. Reducing body mass and/or mass-specific resting metabolic rate (RMR) is one strategy to reduce energetic demands (Deerenberg et al., 1998; Rezende et al., 2006b; Speakman and Selman, 2003). Perrigo and colleagues have shown reduced investment in reproduction by female mice challenged to work for food (Perrigo, 1987; Perrigo and Bronson, 1985).

Experiments by Adage et al. have shown that rats challenged to work for food undergo numerous physiological changes, including a reduction in body mass, blood glucose, and insulin levels, accompanied by increases in insulin sensitivity, adrenocorticotropin hormone (ACTH), and corticosterone level (T. Adage, G. H. Visser and A. J. W. Scheurink, personal communication). In these rats there was large inter-individual variation in the amount of wheel running rats could perform. The ability to maintain body mass during the working period could be predicted from the individual spontaneous wheel-running activity. This raises the intriguing question of whether

spontaneous locomotor activity reflects the physiological capacity of individuals. To address this question, we have exploited the existence of replicate mouse lines that have been selectively bred for high voluntary wheel-running activity (Swallow et al., 1998). We investigated the effects of an increase in foraging effort on behaviour, energy metabolism, body temperature and body composition in both the selected lines and their random-bred control lines. Animals were housed in specialized cages with a running wheel and food dispenser. A computer controlled food rationing as determined by wheel-running activity. With this paradigm, as pioneered by Perrigo and Bronson (Perrigo and Bronson, 1983; Perrigo and Bronson, 1985), we could experimentally vary the wheel-running activity required to obtain a pellet of food. This is intended to mimic variations in the work animals would need to do to secure a living in nature under varying food availability. The present study had two aims: first, to investigate physiological and behavioural responses to high workloads, and second, to investigate whether mice with a high spontaneous level of wheel running would respond differently to the exposed challenge. Because they possess various apparent adaptations for high activity [e.g. elevated maximal oxygen consumption (Rezende et al., 2006a); more symmetrical hindlimb bones (Garland and Freeman, 2005)], we expected mice from the selected line to be more capable of increasing their wheel-running activity without major changes in their physiology and body mass.

Materials and methods

Animals and housing

Outbred Hsd:ICR mice (*Mus domesticus*) selected for high wheel-running activity over 31 generations and their random bred controls were obtained from Theodore Garland, Jr [for selection procedure see Swallow et al. and Garland (Swallow et al., 1998; Garland, 2003)], and a breeding colony (without further selection) was started at the Zoological Laboratory in Haren, The Netherlands. Sixteen male mice, 8 from one of the control lines (C; laboratory designation is line 2) and 8 from one of the selected lines (S; line 7) were used in the experiments. At 4–5 weeks of age, mice were housed individually in cages (30×30×40 cm) equipped with a plastic running wheel (14.5 cm diameter, code 0131; Savic®, Kortrijk, Belgium). They were maintained on a 12:12 L:D cycle (lights on at 08:00 CET). Food [standard rodent chow RMB-H (2181), with a gross energy content of 16.2 kJ g^{-1}; HopeFarms, Woerden, The Netherlands] and water were provided *ad libitum*. Spontaneous wheel-running activity was recorded automatically by a PC-based event recording system (ERS) and stored in 2-min bins. Body mass and food intake were determined throughout the whole experiment at 11:00 each day. When the animals worked for food, pellets (0.045 g per pellet) that were not eaten were removed, counted and deducted from the total number of pellets the mice received. However, small, crumbled and wasted pieces of food (orts) were not removed, and hence represent an uncontrolled, but probably minor (~2%), source of error variance (see Johnson et al., 2001; Koteja et al., 2003). All procedures concerning animal care and treatment were in accordance with the regulations of the ethical committee for the use of experimental animals of the University of Groningen [License DEC 3039(–1)].

Experiment 1: individual maximum workload

All mice were kept for 30–40 days under *ad libitum* food conditions. At 8–9 weeks of age, food was removed and the running wheel was connected to a food dispenser (Med Associates pellet dispenser ENV-203; Sandown Scientific, Hampton, UK) that released a food pellet (45 mg precision food pellets with a gross energy content of 13.4 kJ g^{-1}; Sandown Chemicals, Hampton, Surrey, UK) at a set number of revolutions (General Electric Series 3 Programmable Controller). The number of revolutions per pellet was established for each mouse by dividing its mean spontaneous daily wheel-running activity over the previous week (its baseline wheel-running) by 150. When running at baseline a mouse would thus receive 6.8 g of food (150×0.045), which is similar to the amount of food a mouse on *ad libitum* food would eat. On average, mice had to run 218 (s.d. 54) revolutions per pellet at baseline level. All animals were kept at this level for two days, then the number of revolutions was increased by 15% of the baseline every two days until the animal reached its maximum wheel-running activity. This maximum was defined as the value at the start of a 3-day period of decreasing wheel-running activity. After the maximum was established, animals stayed in the same cages with a running wheel and received *ad libitum* food to allow recovery.

Experiment 2: behavioural and physiological consequences of high workload

Because we did not show any statistically significant differences in the response to workload between control (C) and activity-selected (S) mice in experiment 1 (see Results section), animals from both groups were pooled in experiment 2. These animals will be referred to as ‘Workload mice’ (N=16).

The Workload mice were allowed to recover from experiment 1 for at least four weeks prior to the start of experiment 2. Again, food was taken away and the running wheels were connected to food dispensers *via* the computer system on day zero (t=0). Animals had to work at baseline level for two days, and then over a period of 14 days the workload was increased by equal steps every two days until the workload had increased to 90% of the individual maximal wheel-running activity established in experiment 1. Mice were kept at this level for 10 days and then terminated.

To test whether the Workload mice had sufficiently recovered from experiment 1 and to enable comparisons of body composition an extra control group was used. Mice in this control group were housed in standard cages with a running wheel (15×30×15cm, Macrolon Type II long; UNO Roestvaststaal BV, Zevenaar, The Netherlands) when they were 4-5 weeks old, and received *ad libitum* food [standard

rodent chow RMB-H (2181), HopeFarms] throughout the experiment. The group consisted of three mice from the C line and four from the S line. This group will be referred to as '*Ad-lib* mice' (*N*=7).

Metabolic measurements

In the Workload mice body temperature, daily energy expenditure (DEE) [using the doubly labeled water technique (DLW)] and RMR (indirect calorimetry) were determined twice, once during baseline (day –4 to 0) and once during workload (day 19 to 23, at 90% of maximal workload). In the *Ad-lib* group, DEE and RMR were determined once (at the same age as the working mice during the second measurements).

The protocol for the measurements was as follows. First, mice were weighed on a balance to the nearest 0.1 g and body temperature was measured using a rectal probe inserted to a depth of approximately 10 mm (±0.1°C, NTC type C; Ahlborn, Holzkirchen, Germany) for 15 s. Thereafter we injected the animal with approximately 0.1 g DLW (^{2}H and ^{18}O concentrations of the mixture 37.6% and 58.7%, respectively), allowing an equilibration period of 1 h. The precise dose was quantified by weighing the syringe before and after administration to the nearest 0.0001 g. After puncturing the end of the tail, an 'initial' blood sample was collected and stored in three glass capillary tubes, each filled with approximately 15 μl blood. These capillaries were immediately flame-sealed with a propane torch for later analysis. Thereafter the mouse was returned to its cage. Measurements of body temperatures and injections of DLW were performed in two cohorts of eight mice (Workload) on two consecutive days between 11:00 and 11:30 to minimize the time difference between measurements in different mice. After 48 h a 'final' blood sample was collected as described before, and the animal was weighed again. We collected blood samples of four sentinel mice from our breeding colony that had not been injected with DLW, to assess the natural abundances of ^{2}H and ^{18}O in the body water pools of the animals. Throughout these measurements the Workload mice were working for their food at 90% of their previously observed maximum (experiment 1), and the *Ad-lib* mice had access to a running wheel.

The next day at 12:00, animals were transferred to an eight-channel respirometry system to determine RMR. Mice were put in flow-through boxes (15×10×10 cm) connected to an open-flow respirometry system where oxygen consumption ($\dot{V}_{O_2}$, l h^{-1}) and carbon dioxide production ($\dot{V}_{CO_2}$, l h^{-1}) was measured simultaneously with ambient temperature and activity for 24 h, as described by Oklejewicz et al. (Oklejewicz et al., 1997). In brief, oxygen and carbon dioxide concentration of dried inlet and outlet air (drier: molecular sieve 3 Å; Merck, Damstadt, Germany) from each chamber was measured with a paramagnetic oxygen analyzer (Xentra 4100; Servomex, Crowborough, UK) and carbon dioxide by an infrared gas analyzer (Servomex 1440), respectively. The system recorded the differentials in oxygen and carbon dioxide between dried reference air and dried air from the metabolic cages. Flow rate of inlet air was set at 20 l h^{-1} and measured with a mass-flow controller (Type 5850; Brooks, Rijswijk, The Netherlands). Data were collected every 10 min and automatically stored on a computer. Animals from the Workload groups received ~3 g of food (based on their food intake at that moment) and a piece of apple while in the respirometer. Animals from the other group (*Ad-lib mice*) had *ad libitum* food and a piece of apple.

Metabolic rate (MR, kJ h^{-1}) was calculated using the following equation: $MR=(16.18\times\dot{V}_{O_2})+(5.02\times\dot{V}_{CO_2})$ (Romijn and Lokhorst, 1961). RMR was defined as the lowest value of MR in half-hour running means. RMR in this study thus represents the lowest MR of animals at room temperature (22°C).

Mass spectrometry

The determinations of the ^{2}H/^{1}H and ^{18}O/^{16}O isotope ratios of the blood samples were performed at the Centre for Isotope Research, employing the methods described in detail by Visser and Schekkerman (Visser and Schekkerman, 1999) using an SIRA 10 isotope ratio mass spectrometer. In brief, each capillary was microdistilled in a vacuum line. The ^{18}O/^{16}O isotope ratios were measured in CO_2 gas, which was allowed to equilibrate with the water sample for 48 h at 25°C. The ^{2}H/^{1}H ratios were assessed from H_2 gas, which was produced after passing the water sample over a hot uranium oven. With each batch of samples, we analysed a sample of the diluted dose, and at least three internal laboratory water standards with different enrichments. These standards were also stored in flame-sealed capillaries and were calibrated against IAEA standards. All isotope analyses were run in triplicate.

The rate of CO_2 production (rCO_2, mol d^{-1}) for each animal was calculated with Speakman's equation (Speakman, 1997):

$$rCO_2 = N / 2.078 \times (k_o – k_d) – 0.0062 \times N \times k_d ,$$

where N represents the size of the body water pool (mol) and k_o (d^{-1}) and k_d (d^{-1}) represent the fractional turnover rates of ^{18}O and ^{2}H, respectively, which were calculated using the age-specific background concentrations, and the individual-specific initial and final ^{18}O and ^{2}H concentrations. The value for the amount of body water for each animal was obtained from the carcass analyses. The amounts of body water of the animals at baseline conditions were calculated from the body water *versus* body mass relationship of the seven control animals. Finally, the rCO_2 was converted to energy expenditure, assuming a molar volume of 22.4 l mol^{-1} and an energetic equivalent per l CO_2 based on respiratory quotient (RQ) measurements in our respirometry setup [on average 22 kJ l^{-1} CO_2 (Gessaman and Nagy, 1988)].

Body composition

After the respirometry measurement all animals were euthanized with CO_2 followed by decapitation, and organs were dissected out and weighed to the nearest 0.0001 g. Stomach and intestine were weighed with and without their content. All tissues were stored at –20°C until further analysis. Dry and dry-lean organ masses were determined by drying organs to a constant mass at 103°C, followed by fat extraction

with petroleum ether (Boom BV, Meppel, The Netherlands) in a soxhlet apparatus.

Hormones

Blood samples were taken from the Workload mice from the tail tip during baseline (day –5) and workload (day 18) at 10:00 (one hour prior to daily weighing). Behaviour of the mice was noted prior to sampling, and all mice were at rest. Animals were not anaesthetized and samples were collected within 90 s of initial disturbance. Blood was collected in Eppendorf tubes with EDTA as anticoagulant and kept on ice until it was centrifuged at 2600 ***g*** at 4°C. The supernatant was collected and stored at –80°C. Corticosterone levels were determined using a radioimmunoassay (RIA) kit (Linco Research, Nucli Lab B.V., Ede, The Netherlands).

Data analysis

Statistical analysis was performed using SPSS for Windows (version 14.0). For experiment 1, we applied repeated-measures analysis of variance (ANOVA) with line (C *versus* S) as between-subjects factor and treatment (baseline *versus* workload) as within-subjects factor. For experiment 2, paired *t*-tests were used to test for differences between baseline and workload conditions within the Workload animals, and independent *t*-tests were used to test for differences between *Ad-lib* and Workload animals. All tests were two-tailed and significance was set at $P \leqslant 0.05$.

Results

Experiment 1: maximum workload

Table 1 shows values of wheel-running activity, body mass and absolute and mass-specific food intake in the Workload mice during baseline and at maximum workload. Overall, wheel-running activity did not differ statistically between C and S mice (Table 1, no effect of line). However, as illustrated in Fig. 1, *post-hoc* *t*-tests showed that spontaneous wheel-running activity under baseline conditions was significantly higher in S mice (14.7 km day^{-1}, see Table 1) than in C mice (11.5 km day^{-1}; P=0.05). Body mass and food intake did not differ between C and S mice (Table 1).

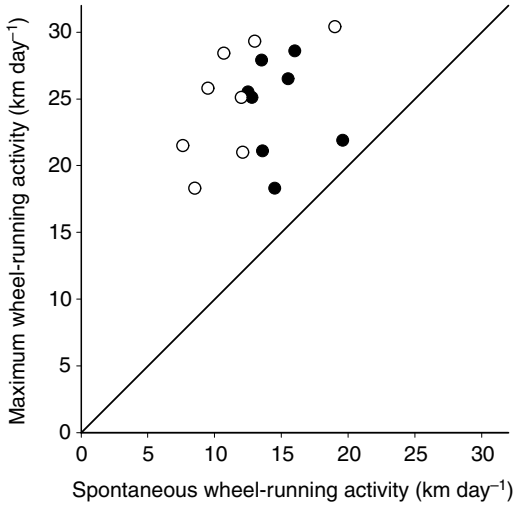

Fig. 1. Relationship between spontaneous wheel-running activity (RWA BL) and maximum wheel-running activity (RWA MX) in control mice (C, open circles) and mice selectively bred for high wheel-running activity (S, closed circles). Linear regression gave the following equations: combining both groups, RWA MX=0.35 RWA BL+20.1 (r^2=0.09, n.s.); for C mice, RWA MX=–0.22 RWA BL+27.6 (r^2=0.02, n.s.) and for S mice, RWA MX=0.84 RWA BL+15.2 (r^2=0.47, n.s.). The line shows where x=y.

When challenged to work for food, all mice increased wheel-running activity (Fig. 1). The maximum level of running did not differ statistically between C and S mice, and was on average 23.3 km day^{-1} in both groups (Table 1). This maximum level was independent of the spontaneous baseline wheel-running activity of the individual mice, as shown in Fig. 1 (Pearson's r=0.3, two-tailed P=0.26). At the maximal

Table 1. *Experiment 1: effects of maximal workload on main characteristics in control (C) and activity-selected mice (S)*

	Baseline		Maximal workload		*P* values			Power of analysis
	C (*N*=8)	S (*N*=8)	C (*N*=8)	S (*N*=8)	d.f.	Line	Treatment	
Wheel-running activity (km day^{-1})	11.5±1.2	14.7±0.8	23.2±1.4	23.4±1.4	1,14	0.29	**<0.001**	0.22/0.24
Body mass (g)	30.9±0.5	30.6±0.5	26.0±0.3	25.5±0.5	1,14	0.82	**<0.001**	0.98/0.99
Food intake (g day^{-1})	5.7±0.1	6.0±0.2	4.6±0.3	4.6±0.2	1,14	0.44	**<0.001**	0.52/0.17
Mass-specific food intake (g g^{-1} day^{-1})	0.20±0.01	1	0.18±0.01	0.18±0.01	1,14	0.38	0.43	0.96/0.54

Wheel-running activity, body mass and food intake during baseline and at maximal workload in activity-selected mice and random-bred controls. Values given are mean ± s.e.m. Repeated-measures ANOVA with line (C *versus* S) as between-subjects factor and treatment (baseline *versus* workload) as within-subjects factor were performed. Interactions between line and treatment was never significant and are therefore not shown in the Table. Significant results are highlighted in bold.

d.f., degrees of freedom; *N*, sample size.

A power analysis for the two-tailed *t*-tests was performed at a fixed effect size of 10% difference between the lines using the Gpower program (Faul and Erdfelder, 1992); we entered mean values and s.e.m. measured for control mice and calculated the power when the mean for the selected mice differed from the controls by 10%. The values in the Table represent the power for the comparison between C and S mice at baseline and workload, respectively.

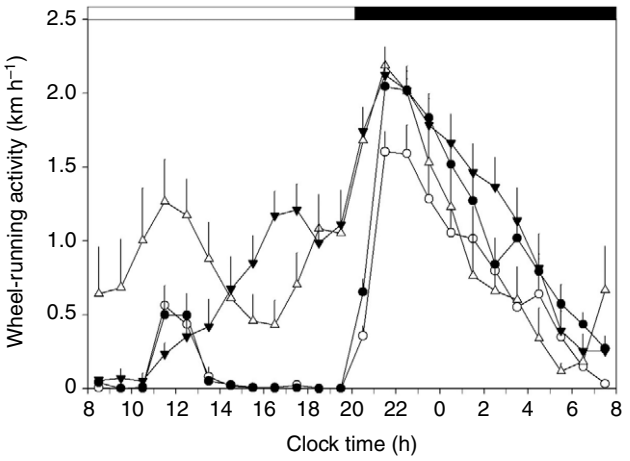

Fig. 2. Circadian pattern of wheel-running activity in control (C, open symbols) and activity-selected mice (S, closed symbols) running spontaneously (circles) or running for food (triangles). Each symbol plots the mean distance ran in hourly bins (e.g. bin 12=from 12:00 until 13:00). Vertical bars are inter-individual s.e.m. The black bar on top represents the dark phase.

level of wheel running, body mass had decreased by approximately 16% and absolute food intake by 20% (significant effect of treatment; see Table 1). Mass-specific food intake did not differ between baseline and workload condition (no effect of treatment, Table 1). No significant interaction effects were seen between line and treatment. C and S mice thus responded similarly to the workload schedule, and both groups showed a similar increase in wheel-running activity and similar decreases in body mass.

Fig. 2 shows the circadian pattern of wheel-running activity during baseline and workload. Under baseline conditions, mice mainly ran in the dark phase. A small peak in wheel-running activity after 11:00 (time of daily measurements) can be observed, probably because of disturbance for daily measurements of body mass and food intake. When challenged to work for food, the period of running was extended and mice started running more during the light phase. It appears that the mice shifted the onset of activity towards the time at which daily measurements took place.

Experiment 2: behavioural and physiological consequences of high workload

Experiment 1 showed no differences in wheel-running activity, body mass or food intake between C and S mice under the high workload conditions. In experiment 2 we therefore pooled data from both groups (Workload mice, N=16) to study the effects of workload on behavioural and physiological traits. Effects of workload were investigated by comparing the baseline condition (*ad libitum* food) to the high workload condition (wheels attached to food dispenser) within these mice (using paired t-tests). For comparison of body composition, however, an additional control group of seven age-matched animals housed with a wheel and *ad libitum* food was added (*Ad-lib* group). This extra control group also enabled us to determine whether the Workload mice had sufficiently recovered from experiment 1 before the start of experiment 2.

Development of body mass, food intake, and wheel-running activity at sub-maximal workload

For daily measurements (body mass, food intake, and wheel-running activity) we calculated a baseline and workload value that was the mean over one week (see Table 2). For the baseline condition, this was the week prior to the start of the training, and for the workload the week started when the animals were on a maximal workload for 2 days.

To determine whether the animals had recovered sufficiently from experiment 1, we first compared baseline data (Workload

Table 2. *Experiment 2: main characteristics of ad-libitum-fed animals and Workload animals at baseline or workload conditions*

	Ad-lib animals (N=7)	Workload animals (N=16)	
		Baseline	Workload
Wheel-running activity (km day^{-1})	7.7±1.3	10.2±0.9[b]	20.2±1.5
Body mass (g)	34.2±0.8	34.6±0.5[b]	28.2±0.5
Food intake (g day^{-1})	4.3±0.4	6.4±0.2[a,b]	4.0±0.2
Mass-specific food intake (g g^{-1} day^{-1})	0.13±0.01	0.19±0.01[a,b]	0.14±0.01
RMR (kJ day^{-1})	49.5±1.9	49.3±1.2[b]	27.4±1.8
Mass-specific RMR (kJ g^{-1} day^{-1})	1.45±0.05	1.43±0.03[b]	0.98±0.05
DEE (kJ day^{-1})	62.6±2.9	72.3±1.7[a,b]	60.0±1.7
Mass-specific DEE (kJ g^{-1} day^{-1})	1.83±0.10	2.09±0.04[a,b]	2.25±0.07
Body temperature (°C)	–	36.6±0.4[b]	35.4±0.8
Corticosterone ($\times10^3$ ng ml^{-1})	–	15±5[b]	222±47

Wheel-running activity, body mass, food intake, resting metabolic rate (RMR), daily energy expenditure (DEE), body temperature and corticosterone level are shown for Workload animals under baseline and workload conditions and for *ad libitum*-fed mice.

Values are mean ± s.e.m. One control animal died during the respirometry measurements and data on RMR were thus not available.

[a]Significant difference between *Ad-lib* and Workload mice at baseline (independent t-test, P<0.05). [b]Significant difference within the Workload group between baseline and workload conditions (paired t-test, P<0.05).

group) with data on animals in the *Ad-lib* group of the same age using independent *t*-tests (see Table 2). *Ad-lib* and Workload mice under baseline conditions did not systematically differ in body mass or wheel-running activity (see Fig. 3, triangles, and Table 2). Food intake was slightly lower in *Ad-lib* mice than in Workload mice (4.3 *versus* 6.3 g day^{-1}). These results indicated that mice had recovered sufficiently from the preliminary workload experiment and subsequently the new workload scheme was started.

Fig. 3 shows the changes in body mass and food intake that occurred in the Workload mice when put on a workload schedule. On day 0 wheels were attached to the food dispensers and the foraging effort was increased over 14 days up to 90% of the previously observed maximum for each mouse (training period). Mice were kept at this level for 10 days (workload period). Wheel-running activity showed a slight decrease just before the start of the training, which can probably be attributed to the manipulations done at this time (DLW injections). Wheel-running activity increased steadily during the training period and reached a plateau of approximately 20 km d^{-1} at the highest workload (90% of maximum workload). In the Workload mice, body mass decreased significantly, with approximately 20% from 34.6 to 28.4 g, and remained at this level from day 20 onwards. Both absolute and mass-specific food intake decreased by approximately 30% at 90% workload compared with baseline. Wheel-running activity approximately doubled at high workload in the Workload mice (see Fig. 3 and Table 2).

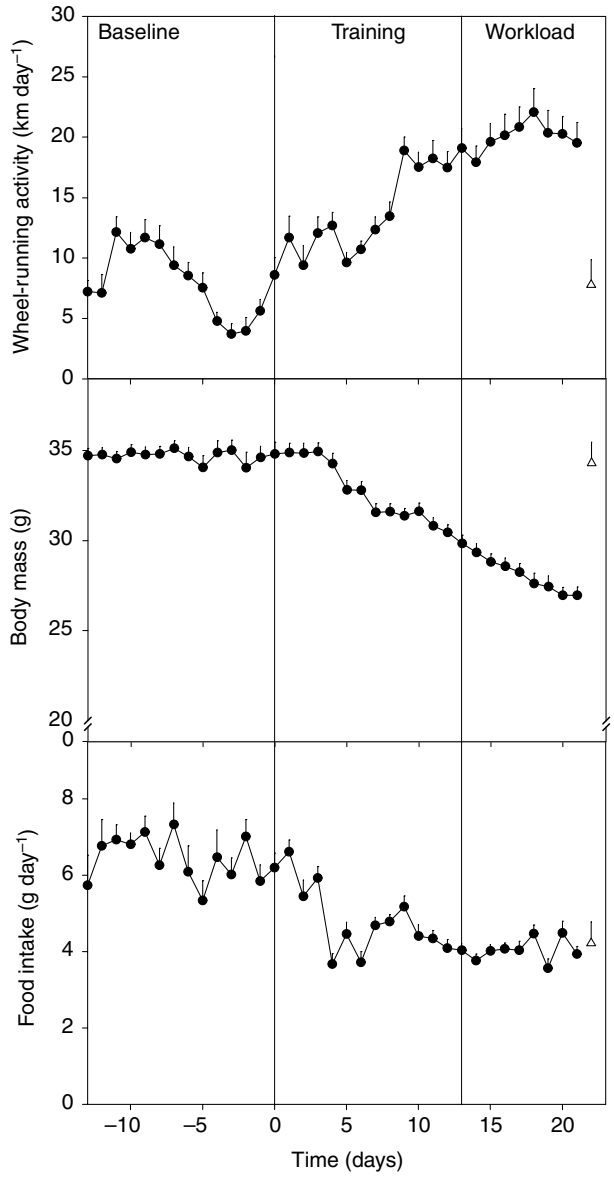

Fig. 3. Development of wheel-running activity, body mass and food intake during training and at a workload of 90% from the maximal capacity in Workload animals (C and S groups pooled). Spontaneous wheel-running activity is shown for the 2 weeks prior to the training period (day 0). Circles show the development of the different variables during the experiment in the Workload animals, and triangles represent mean values for mice in the *Ad-lib* group.

We calculated the mean time spent running by adding up all the 2-min intervals in which running occurred per day, and the maximum speed the mice ran (maximum distance covered per 2-min interval). This was done during baseline and workload to determine which strategy animals used to increase their wheel-running activity. During baseline, time spent running was 5.9 h (s.d. 1.8), but this almost doubled to 11.5 h (s.d. 2.0) during workload. Maximum running speeds were 4.7 km h^{-1} (s.d. 0.8) and 6.3 km h^{-1} (s.d. 0.5) in baseline and workload phases, respectively (paired *t*-test; $P<0.001$ for both). Mice thus increased both time spent running (+94%) and maximum running speed (+34%).

Multiple regression analysis showed that food intake was significantly, positively predicted by both body mass and wheel-running activity at baseline (multiple regression: $r^2=0.49$, $P=0.012$; body mass, $P=0.018$; wheel-running activity, $P=0.067$), as well as during the high workload experiment (multiple regression: $r^2=0.58$, $P=0.004$; body mass, $P=0.0012$; wheel-running activity, $P=0.002$).

Metabolic rate

Metabolic rate of the Workload animals was measured under baseline and workload conditions (Table 2). First, we compared RMR and DEE between *Ad-lib* animals and Workload animals at baseline (see Table 2). No significant differences were found for RMR, but DEE was significantly lower in the *Ad-lib*-fed mice, which might be because of the slightly smaller cages they were housed in. Second, we compared RMR and DEE under baseline and workload conditions within the Workload group. At 90% of maximum workload, mice decreased RMR by approximately 50%, from a mean of 49.3 kJ d^{-1} to 27.4 kJ d^{-1}. The reduction in mass-specific RMR was approximately one-third, from 1.43 to 0.98 kJ g^{-1} d^{-1}. Both differences were statistically significant. Workload also influenced absolute and mass-specific DEE. Absolute DEE decreased on average from 72.3 to 60.0 kJ d^{-1} at high workload, but mass-specific DEE slightly increased from 2.09 to 2.25 kJ d^{-1}. Both differences were statistically

significant (Table 2). Looking at individual variation, all mice except one individual exhibited a decrease in DEE during workload (whole-animal values).

We estimated the cost of activity (ACT, in kJ day^{-1}) by deducting RMR from DEE (ACT was 23.0 and 32.6 kJ d^{-1} at baseline and workload, respectively), and divided this by the amount of wheel running to estimate the costs per km. Costs of running were 2.3 kJ km^{-1} (s.d. 1.6) and 1.6 kJ km^{-1} (s.d. 0.3) at baseline and workload, respectively. This difference was significant (paired *t*-test, two-tailed, P=0.026).

It is well-known that metabolic rates (RMR and DEE) are positively associated with body mass, and under baseline conditions this relationship was obvious in all mice, based on bivariate relationships (open symbols in Fig. 4A; Table 3). However, when working for food there was no longer a statistically significant relationship between body mass and metabolic rates (closed symbols in Fig. 4A; Table 4). We also performed multiple regression analyses with body mass and wheel-running activity as independent predictors of RMR or DEE. At baseline, the models including both body mass and wheel-running activity were significant (r^2=0.48, P=0.015), but only body mass (P=0.007) and not wheel-running activity (P=0.148) significantly predicted RMR. The same was true for the relationship with DEE (r^2=0.43, P=0.025; body mass, P=0.008; wheel-running activity, P=0.785). Body mass alone explained more of the variation in RMR and DEE than models that included wheel-running activity (see Table 3).

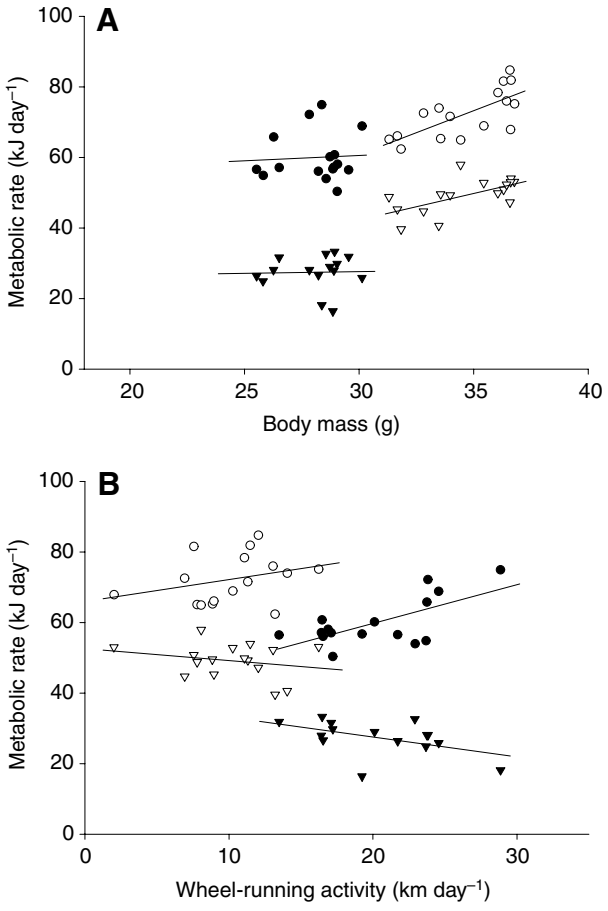

Fig. 4. Relationship between body mass and metabolic rates (A) and between wheel-running activity and metabolic rates (B) at baseline (open symbols) and workload (closed symbols) conditions in Workload animals. Triangles represent the RMR and circles represent DEE. Regression lines for all relationships are drawn. For equations of the regression lines, r^2 and P values, see Table 4. Results of multiple regressions are presented in the text.

At high workload, metabolic rates were better predicted by the amount of wheel-running activity than by body mass (see Fig. 4B and Table 3). Multiple regressions for DEE or RMR with body mass and wheel-running activity were not significant (RMR: r^2=0.25, P=0.182; body mass, P=0.709; wheel-running activity, P=0.071; and for DEE: r^2=0.25, P=0.158; body mass, P=0.241; wheel-running activity, P=0.073). As shown in Table 4, wheel-running activity alone did significantly predict DEE (P=0.005), and approached significance for predicting RMR (P=0.065). RMR was negatively related to wheel-running activity, whereas DEE was positively related to wheel-running activity at workload. The animals that ran the most thus decreased their RMR the most, while increasing DEE. RMR and DEE at baseline did not relate to RMR and DEE at workload.

Energy balance

Fig. 5 shows the energy budget of Workload mice at baseline and workload calculated over the days when DEE was measured in these mice. The figure shows the various components of the energy budget; gross energy intake (GEI), metabolisable energy intake (MEI) and DEE divided into RMR and energy spent on activity (ACT). GEI was calculated on the basis of the measured food intake and was 97.4 and 53.6 kJ d^{-1} in mice under baseline and workload conditions, respectively (see Materials and methods, for gross energy content of the food). Animals are not 100% efficient in metabolising their food and the actual amount of energy animals take out of their food can only be calculated when digestive efficiency and the amount of energy lost in the urine has been measured as well. Previous studies have shown a digestive efficiency of 79.1% in *ad libitum*-fed mice, including loss of energy in urine (Hambly and Speakman, 2005). Under the assumption that workload did not alter digestive efficiency, MEI at baseline and workload was estimated using a digestive efficiency of 79.1%. Based on these values, we can see whether animals were in a positive or negative energy balance. It is clear from this picture that at high workload the proportion of energy used for RMR was strongly decreased and the energy available for activity had increased. At high workloads there was a negative energy budget of –17.7 kJ d^{-1} (or –0.74 kJ g^{-1} d^{-1}), and the extra energy needed was obtained by reducing body mass by 0.8 g on average. During baseline the energy budget was positive, +4.7 kJ d^{-1} (or +0.15 kJ g^{-1} d^{-1}), and animals gained 1.0 g body mass over the course of the measurements. Even after assuming an unlikely digestive efficiency of 100% in the Workload animals, the energy budget would still be negative (–6.4 kJ).

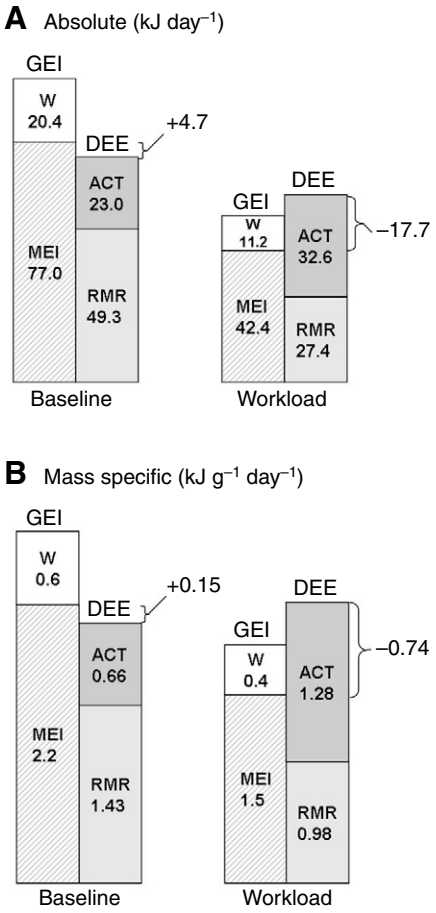

Fig. 5. Energy budget of Workload mice during baseline and workload conditions. Panel A shows the absolute values and panel B the mass-specific values. To determine the energy balance we used measures of resting metabolic rate (RMR, light-grey bars) and daily energy expenditure (DEE). Energy for activity (ACT, darker grey bars) was calculated by deducting RMR from DEE. In addition, the gross energy intake (GEI) was calculated on the basis of the absolute food intake during the DLW measurements. Metabolisable energy intake (MEI, striped bars) was then calculated from GEI, assuming that digestive efficiency together with energy lost in the urine was 79.1% (Hambly and Speakman, 2005). The white bars represent the energetic value of the food that is not metabolized (GEI–MEI=Waste, W). The numbers in the bars represent the amount of energy (either in kJ d^{-1} or in kJ g^{-1} d^{-1}) spent on each part of the energy budget. The bracket shows the surplus energy available to the animals for growth.

Body composition

We compared data from the animals in the Workload group with the animals in the *Ad-lib* group using independent *t*-tests to investigate the effects of workload on body composition (see Table 4). Body mass, total dry lean, and fat content were strongly decreased in animals in the Workload group. Fat content decreased the most, by 70%, from 3.1 to 0.9 g. Dry lean organ masses were significantly decreased in all organs of working animals compared with *Ad-lib* animals, except for the brain, stomach and lungs that showed no difference, and the intestines that showed a significant increase in dry lean mass.

Table 3. *Linear regressions of metabolic rates (RMR and DEE) on body mass or on wheel-running activity in Workload mice*

Linear regression	Slope	Intercept	r^2	*P*
Baseline				
Body mass *versus* RMR	1.52	–3.3	0.38	0.011
Body mass *versus* DEE	2.48	–13.5	0.5	0.002
Wheel running *versus* RMR	–0.35	52.9	0.06	0.370
Wheel running *versus* DEE	0.61	66.2	0.09	0.260
Workload				
Body mass *versus* RMR	0.13	23.8	0.01	0.390
Body mass *versus* DEE	0.36	50	0.05	0.790
Wheel running *versus* RMR	–0.56	38.8	0.24	0.065
Wheel running *versus* DEE	1.10	37.7	0.41	0.005

Multiple regressions showed that at baseline RMR and DEE were better predicted by body mass alone, and at workload by wheel-running activity alone. Results of multiple regressions with both body mass and wheel-running activity as independent predictors of RMR or DEE are described in the text. See also Fig. 4.

Fat content also decreased significantly in most organs (except for the heart), with the largest decrease in skin (81%) and the lowest in the brain (10%).

We also calculated mass-specific organ masses (organ mass as a fraction of total fresh body mass) to enable more appropriate comparisons of groups that differ in body mass (data not shown). In these analyses, total fat content and fat content of all organs (except for heart) still showed a significant decrease. Total mass-specific dry lean mass did not differ between *Ad-lib* and Workload animals anymore; dry lean mass did significantly decrease in liver, kidney, skin and the remainder of the carcass, but it increased significantly in brain, stomach, intestine and lung.

The total fat content of the mice could be negatively predicted by the amount of wheel-running activity at workload (r=–0.67, P=0.006).

Body temperature and plasma corticosterone

Body temperature of the Workload animals was measured in the light phase under baseline and workload conditions (see Table 2). Three out of 16 mice under workload conditions had extremely low body temperatures at the time of measurement (32.2, 32.5 and 26.8°C), but no significant differences were found within the Workload mice between baseline or workload conditions. Plasma corticosterone levels were strongly affected by treatment. At high workload, corticosterone levels were approximately 15-times increased (see Table 2). Individual variation in body temperature or plasma corticosterone did not correlate with wheel-running activity (data not shown).

Discussion

Wheel-running activity was approximately 30% higher in activity-seleced mice (S) compared with their random-bred controls (C) under baseline conditions (see experiment 1), but,

unexpectedly, wheel-running activity did not differ between the lines at high workload. S mice show several adaptations to their high wheel-running activity [e.g. elevated maximal oxygen consumption (Rezende et al., 2006a) and more symmetrical hindlimb bones (Garland and Freeman, 2005)], but these adaptations did not result in a higher capacity to run when working for food in the one S line as compared with the one C line that we studied. In our second experiment we therefore did not focus on line effects, but on effects of high workload on several physiological and behavioural traits.

Challenging mice to work for food to mimic low food availability resulted in several physiological and behavioural changes that may be adaptive. All animals increased wheel-running activity by approximately 100%. This was mainly accomplished by spending more time running (including during the light phase), but running speed also increased. A shift in activity patterns towards the day in response to workload was shown before in *Mus musculus* (Perrigo, 1987). The increase in wheel-running activity was not sufficient to maintain adequate food intake, and body mass decreased (Fig. 3).

A detailed look at the body composition of the Workload mice showed that the reduction in body mass was mainly caused by a reduction in fat mass. Total fat content was reduced by ~70% in Workload mice compared with mice in the *Ad-lib* group. Fat content of all organs (except for the heart) reduced significantly, with the most pronounced decreases in subcutaneous and intra-peritoneal fat and the smallest decrease in the brain. Similarly, dry lean mass of the brain was not significantly reduced; mass-specific dry lean mass of the brain even increased in the Workload group. The brain is important for the central regulation of bodily functions and is apparently protected in times of scarcity. A similar result was found in food-restricted rats, where brain mass was unaffected, but heart, kidney and liver mass decreased (Greenberg and Boozer, 2000). Total mass-specific dry lean mass was similar in *Ad-lib* and Workload mice, but the distribution of dry lean mass over the body did change under high workload conditions. In liver, kidney, skin and the remainder of the carcass, mass-specific dry lean mass was decreased, whereas it was increased in lung, stomach and intestine. The increase in intestine mass and stomach mass could indicate that animals increased their digestive efficiency to extract more energy from a gram of food. Several studies have shown that changes in gut morphology do not generally increase digestive efficiency (Corp et al., 1997; Hammond et al., 1996), and measurements of digestive efficiency are thus necessary. Mice could also have ingested their faeces (coprophagy) to increase their food efficiency even more. Further studies are necessary to test these hypotheses.

The strong reduction in fat content without a major change in dry lean mass is in agreement with observations by Perrigo and Bronson in pre-pubertal female mice (Perrigo and Bronson, 1983). In their study, fat depots remained undiminished or above

Table 4. *Body composition of ad-libitum-fed mice and mice working for food*

Variable (g)	*Ad-lib* animals (*N*=7)	Workload animals (*N*=15)	Difference (%)	Independent *t*-test	
				t	*P*
Body mass	31.4±0.9	25.9±0.4	–17	–6.8	<0.001
Dl mass	8.1±0.2	6.6±0.1	–19	–9.9	<0.001
Fat content	3.13±0.35	0.94±0.09	–70	–8.2	<0.001
Dl heart	0.04±0.001	0.03±0.001	–29	–4.8	<0.001
Dl liver	0.48±0.03	0.30±0.02	–37	–5.0	<0.001
Dl kidney	0.13±0.01	0.10±0.001	–29	–4.0	0.001
Dl brain	0.08±0.001	0.08±0.001	–3	–1.3	0.23
Dl stomach	0.04±0.001	0.04±0.001	+5	0.9	0.36
Dl intestines	0.29±0.01	0.34±0.01	+18	4.5	<0.001
Dl lung	0.04±0.001	0.04±0.001	+3	0.6	0.59
Dl skin	1.50±0.04	1.26±0.02	–16	–6.0	<0.001
Dl rest	5.48±0.12	4.35±0.05	–21	–10.6	<0.001
Fat heart	0.006±0.001	0.005±0.001	–18	–1.1	0.30
Fat liver	0.048±0.009	0.027±0.003	–43	–2.8	0.011
Fat kidney	0.026±0.003	0.006±0.001	–75	–7.7	<0.001
Fat brain	0.047±0.001	0.043±0.001	–10	–3.7	0.002
Fat stomach	0.008±0.001	0.005±0.001	–36	–4.2	<0.001
Fat intestines	0.070±0.010	0.039±0.002	–44	–4.2	<0.001
Fat lung	0.008±0.001	0.004±0.001	–51	–6.7	<0.001
Fat skin	0.85±0.120	0.16±0.028	–81	–7.6	<0.001
Fat rest	2.07±0.240	0.65±0.071	–69	–7.4	<0.001

Values are mean ± s.e.m. Total dry lean (dl) mass, fat content and the dl mass and fat mass of separate organs are shown for Workload and *Ad-lib* mice. The body mass shown is the total body mass minus the gut and intestine content at time of death. One mouse died during the second respirometry measurement in the Workload group. Difference (%) shows the change in mass between *Ad-lib* and Workload animals. Independent *t*-tests were performed to test for differences between groups and the results are shown in the Table.

control levels over a wide range of forced activity, even when accompanied by a moderate decrease in food intake, but at the maximum requirement of 225 revolutions per pellet (comparable to our conditions) females accumulated less body fat than *ad libitum*-fed animals. Studies on food restriction in sedentary rodents show contrasting results on body composition changes, with greater use of fat mass than dry lean mass [rats (Greenberg and Boozer, 2000)], defense of fat mass and reduction of dry lean mass [mice (Hambly and Speakman, 2005)] or no differential use of the different components [rats (Selman et al., 2005)].

Corticosterone levels were increased at high workload and were comparable to the values reported in response to restraint stress in male mice of this strain (Malisch et al., 2007). Baseline values were slightly lower than the ones reported in that study. We did not show a relationship between wheel-running activity (over 24 h) and corticosterone or body temperature. Wheel-running activity in the 10–20 min prior to measurements has been shown to correlate positively with both body temperature (Rhodes et al., 2000) and plasma corticosterone (Girard and Garland, 2002) in these lines of mice. Corticosterone levels also increase in mice and other mammals when they run on a motorized treadmill (Coleman et al., 1998).

Selective breeding for high spontaneous wheel-running activity did not affect the response to a workload challenge, at least based on our comparison of one of the four selected lines with one of the four control lines (see Swallow et al., 1998). Control (C) and activity-selected (S) mice did not differ with respect to their maximum wheel-running activity on a high workload (~23 km d^{-1}; Table 1), and both groups showed similar decreases in food intake and body mass at the maximum workload. Also, spontaneous wheel-running activity at baseline did not predict wheel-running activity at workload (Fig. 1). These results are in contrast to a similar study in rats, *Rattus norvegicus* (T. Adage, G. H. Visser and A. J. W. Scheurink, personal communication). Based on measurements of spontaneous wheel-running activity, they divided female Wistar rats from a single population into high or low spontaneous runners. Animals with high baseline running activity coped better on a workload schedule than rats with low spontaneous levels of wheel-running activity, and the former could also increase their wheel-running activity more. The rats with low spontaneous levels of activity markedly decreased in body mass, whereas rats that had high levels of spontaneous wheel running maintained body mass at the same workload level. The discrepancy between our study and the study of Adage et al. (T. Adage, G. H. Visser and A. J. W. Scheurink, personal communication) may represent differences between mice and rats in the regulation of wheel-running activity and body mass, and may also depend on differences in motivation to run. The rats were of similar age (3–4 months) to our mice and because both have similar lifespans, age was probably not a factor.

Resting metabolic rates and, to a lesser extent, daily energy expenditure showed a strong reduction under workload conditions (~50%), an effect that has been shown in several studies manipulating workload; in birds (Bautista et al., 1998; Deerenberg et al., 1998; Wiersma and Verhulst, 2005), hamsters, *Phodopus sungorus* (Day and Bartness, 2001), and mice (Perrigo, 1987); for a summary see table 4 in Wiersma and Verhulst (Wiersma and Verhulst, 2005). In another study, an increase in DEE has been shown (Wiersma et al., 2005), but that study used a variable- rather than the fixed-reward ratio we used in this study. With increasing wheel-running activity, RMR decreased and DEE increased, but DEE was lower under workload conditions than when animals were running spontaneously at a lower level. In principle, mice had unlimited access to food, but they stopped foraging at a point where their food intake was lower than the food intake of animals that had immediate access to food. Instead of increasing their food intake, animals compensated for the increased costs of activity by decreasing RMR. This may indicate the presence of constraints that prevent animals from increasing their activity further (see also Garland, 2003; Rhodes et al., 2005). First, the capacity for sustained, endurance-type activity can be a limiting factor. Second, time can be a limiting factor, and animals did extend their activity into the light phase on the workload (Fig. 2), thus leaving less time to rest and sleep. All animals need to sleep to survive (Everson, 1995), and this may have limited the time mice had left to run. However, levels of running were much lower during the day than during the night, and animals only spent ~12 h continuously running at high workloads, which would seem to leave enough time for rest. Third, digestive constraints could limit the intake of extra food. Total food intake was reduced at high workload compared with the baseline condition, and it is thus not likely that digestive constraints were at work in our mice. Moreover, when cold-exposed, these mice can increase their food intake by much greater amounts (Koteja et al., 2001) than were ever exhibited in the present study. Another possible constraint is metabolic. When we looked at mass-specific metabolic rates, RMR was reduced in mice at high workload, but DEE was slightly increased. Several lines of evidence indicate that maximum metabolic rates are limited by the intrinsic physiology of the animal (Daan et al., 1990; Drent and Daan, 1980; Hammond and Diamond, 1997; Speakman and Krol, 2005). It has been suggested that this upper sustainable limit is related to basal metabolic rate (BMR) such that a limit is imposed at 4-7×BMR (Daan et al., 1990; Drent and Daan, 1980; Hammond and Diamond, 1997). When animals reach this upper limit they can no longer increase their activity (energy expenditure) to obtain more food. Factors involved in causing these limits may include central limits associated with the energy-supplying machinery (central limitations hypothesis), peripheral limits associated with the energy-consuming machinery (peripheral limitation hypothesis), or a combination of both (Hammond and Diamond, 1997). In running mice, central limits may, for instance, include the ability to digest food (see above) or the capacity of lungs to take up oxygen and exhale carbon dioxide (see also Rezende et al., 2006a). Peripheral limits may include the capacity of skeletal muscles. An alternate hypothesis suggests that the maximal capacity of animals to dissipate heat generated as a byproduct of, for example, processing food and producing milk may be a limiting factor (heat-dissipation hypothesis) (Krol et al., 2003).

The maximum sustainable level of energy expenditure in laboratory mice subjected to forced exercise (*Mus musculus*) has been measured at 3.6×BMR; see table 2 in Hammond and Diamond (Hammond and Diamond, 1997). Our mice were working at 3.7×BMR [assuming an estimated BMR of 0.61 kJ g^{-1} d^{-1} in the Workload mice; based on the RMR measured at 22°C and earlier measurements in this strain of mice at thermoneutrality, 30°C (Vaanholt et al., 2007)], and thus close to their maximal sustainable rate. Studies investigating maximal sustainable rates imposed by other factors than exercise, such as cold exposure and lactation, have shown that mice are capable of even higher rates of energy expenditure. Cold-exposed mice attained values as high as 4.8×BMR (Hammond and Diamond, 1997), and during lactation (also in combination with cold exposure) the sustained energy intakes in mice varied from 6.1 to 9.4×BMR (Hammond and Diamond, 1997; Johnson and Speakman, 2001; Krol et al., 2003). Differences in peripheral limitations, i.e. milk production in lactation *versus* muscle capacity in exercise and/or the capacity to dissipate heat, may explain differences between the different conditions. Our mice thus probably did not increase activity further because they were working close to their maximal sustainable rate. Given that body mass stabilized at the end of the workload period (Fig. 3), animals were probably close to reaching a new energetic balance, similar to what is seen in calorically restricted animals (Hambly and Speakman, 2005; Holloszy and Schechtman, 1991).

To compensate for the experimentally manipulated increase in energy expended on activity, animals reduced RMR. The mice that ran the most showed the greatest decrease in RMR. How could they have accomplished this? First, reducing body mass reduces whole-animal RMR (Deerenberg et al., 1998; Speakman and Selman, 2003). However, the reduction in RMR observed in the present study was much greater than expected based on changes in body mass alone, and at the high workload body mass did not significantly correlate with RMR. As proposed by Rezende et al. (Rezende et al., 2006b), in the lines of mice selectively bred for high running, lowering of body mass may be a way to keep whole-animal energy costs of activity relatively low and selective breeding causes total running distance to increase. Similarly, in animals forced to work for food, lowering body mass may be a way to decrease costs of running and/or maintenance costs. Indeed, when we calculated the energy spent per km at baseline and workload condition, a reduction in whole-animal running costs of approximately 35% was found. The cost of transport (COT) estimated here (2.3 kJ km^{-1}) is much higher than that reported previously (~1.2 kJ km^{-1}) for these mice (Koteja et al., 1999; Rezende et al., 2006b; Vaanholt et al., 2007). This discrepancy occurs because in this study we did not calculate COT based on the slope of the regression between running speed and energy expenditure, but instead made a crude estimate of COT by dividing ACT by the amount of wheel running.

Animals also could have saved energy by reducing behaviours other than wheel-running activity, such as grooming or exploration, or they may have compensated by saving on maintenance processes. It has, for instance, been shown that zebra finches in energetically demanding situations refrain from mounting an immunological response to a novel challenge (Deerenberg et al., 1997) and that they invest less in regrowing feathers (Wiersma and Verhulst, 2005). Further research is necessary to determine whether similar effects may have occurred in our mice. Hypothermia, as we saw in several mice, and that has been reported in previous experiments manipulating foraging effort (Perrigo and Bronson, 1983) and in food-restricted animals [birds (Daan et al., 1989) and mice (Gelegen et al., 2006; Rikke et al., 2003)], may also have contributed to the strong reduction in RMR. In the present study, mice were housed at 22°C, which is well below the lower critical temperature of mice; thermoregulatory costs could have been lowered even more by substituting thermoregulatory heat production for heat generated by activity. However, a previous study of these mice did not show substitution of thermoregulatory heat for heat generated by voluntary activity (Vaanholt et al., 2007). Lowering body temperature can be beneficial to save energy, but lowering body temperature may also impose a trade-off. When body temperature gets below the optimal temperature for enzymatic activity, protein turnover and/or cellular turnover in general decelerates, causing reduced repair of cellular damage or a reduction in immunological defense (Deerenberg et al., 1997). In addition, reduced body temperature may lower locomotor performance (Bennett, 1990) and impair various other physiological rate processes.

In summary, challenging mice to work for food resulted in several physiological changes. Mice readily increased wheel-running activity when they had to work for food, but they did not maintain food intake, and body mass subsequently decreased (mainly by a reduction in fat mass). Animals were working close to their highest maximal sustainable rate at 3.7×BMR. Mice compensated for the increased energetic requirements by decreasing RMR. The physiological responses were independent of inter-individual variation in spontaneous wheel-running activity, but wheel-running at the high workload was negatively related to RMR. The more they ran, the lower their RMR became. DEE showed an opposite relationship.

The authors thank Gerard Overkamp for expert technical assistance with the workload equipment and Berthe Verstappen for performing the isotope analyses. Peter Meerlo enabled us to do the corticosterone measurements. We thank Kristin Schubert and two anonymous reviewers for their help in improving earlier versions of the manuscript. T.G. was supported by US National Science Foundation grant IOB-0543429. S.D. was supported by the European 6th Framework Integrated Program EUCLOCK.

References

Bautista, L. M., Tinbergen, J., Wiersma, P. and Kacelnik, A. (1998). Optimal foraging and beyond: How starlings cope with changes in food availability. *Am. Nat.* **152**, 543-561.

Bennett, A. F. (1990). Thermal dependence of locomotor capacity. *Am. J. Physiol.* **259**, R253-R258.
Bronson, F. H. (1987). Susceptibility of the fat reserves of mice to natural challenges. *J. Comp. Physiol. B* **157**, 551-554.
Carbone, C. (2005). How far do animals go? Determinants of day range in mammals. *Am. Nat.* **165**, 290-297.
Coleman, M. A., Garland, T., Jr, Marler, C. A., Newton, S. S., Swallow, J. G. and Carter, P. A. (1998). Glucocorticoid response to forced exercise in laboratory house mice (Mus domesticus). *Physiol. Behav.* **63**, 279-285.
Corp, N., Gorman, M. L. and Speakman, J. R. (1997). Apparent absorption efficiency and gut morphometry of wood mice, Apodemus sylvaticus, from two distinct populations with different diets. *Physiol. Zool.* **70**, 610-614.
Daan, S., Masman, D., Strijkstra, A. and Verhulst, S. (1989). Intraspecific allometry of basal metabolic rate: relations with body size, temperature, composition and circadian phase in the Kestrel, *Falco tinnuculus*. *J. Biol. Rhythms* **4**, 267-283.
Daan, S., Masman, D. and Groenewold, A. (1990). Avian basal metabolic rates: their association with body composition and energy expenditure in nature. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **259**, R333-R340.
Day, D. E. and Bartness, T. J. (2001). Effects of foraging effort on body fat and food hoarding in Siberian hamsters. *J. Exp. Zool.* **289**, 162-171.
Deerenberg, C., Arpanius, V., Daan, S. and Bos, N. (1997). Reproductive effort decreases antibody responsiveness. *Proc. R. Soc. Lond. B Biol. Sci.* **264**, 1021-1029.
Deerenberg, C., Overkamp, G. J. F., Visser, G. H. and Daan, S. (1998). Compensation in resting metabolism for experimentally increased activity. *J. Comp. Physiol. B* **168**, 507-512.
Drent, R. and Daan, S. (1980). The prudent parent: energetic adjustments in avian breeding. *Ardea* **68**, 225-252.
Everson, C. A. (1995). Functional consequences of sustained sleep deprivation in the rat. *Behav. Brain Res.* **69**, 43-54.
Faul, F. and Erdfelder, E. (1992). *GPower: A Priori, Post-Hoc, and Compromise Power Analyses for MS-DOS*. Bonn, FRG: Dept of Physchology, Bonn University.
Garland, T., Jr (1983). Scaling the ecological cost of transport to body-mass in terrestrial mammals. *Am. Nat.* **121**, 571-587.
Garland, T., Jr (2003). Selection experiments: an under-utilized tool in biomechanics and organismal biology. In *Vertebrate Biomechanics and Evolution* (ed. V. L. Bels, J.-P. Gasc and A. Casinos), pp. 23-56. Oxford: BIOS Scientific Publishers.
Garland, T., Jr and Freeman, P. W. (2005). Selective breeding for high endurance running increases hindlimb symmetry. *Evol. Int. J. Org. Evol.* **59**, 1851-1854.
Gelegen, C., Collier, D. A., Campbell, I. C., Oppelaar, H. and Kas, M. J. (2006). Behavioral, physiological, and molecular differences in response to dietary restriction in three inbred mouse strains. *Am. J. Physiol. Endocrinol. Metab.* **291**, E574-E581.
Gessaman, J. A. and Nagy, K. A. (1988). Energy metabolism: errors in gas-exchange conversion factors. *Physiol. Zool.* **61**, 507-513.
Girard, I. and Garland, T., Jr (2002). Plasma corticosterone response to acute and chronic voluntary exercise in female house mice. *J. Appl. Physiol.* **92**, 1553-1561.
Goszczynski, J. (1986). Locomotor activity of terrestrial predators and its consequences. *Acta Theriol.* **31**, 79-95.
Greenberg, J. A. and Boozer, C. N. (2000). Metabolic mass, metabolic rate, caloric restriction and aging in male Fischer 344 rats. *Mech. Ageing Dev.* **113**, 37-48.
Hambly, C. and Speakman, J. R. (2005). Contribution of different mechanisms to compensation for energy restriction in the mouse. *Obes. Res.* **13**, 1548-1557.
Hammond, K. A. and Diamond, J. (1997). Maximal sustained energy budgets in humans and animals. *Nature* **386**, 457-462.
Hammond, K. A., Lam, M., Lloyd, K. C. and Diamond, J. (1996). Simultaneous manipulation of intestinal capacities and nutrient loads in mice. *Am. J. Physiol.* **271**, G969-G979.
Holloszy, J. O. and Schechtman, K. B. (1991). Interaction between exercise and food restriction: effects on longevity of male rats. *J. Appl. Physiol.* **70**, 1529-1535.
Johnson, M. S. and Speakman, J. R. (2001). Limits to sustained energy intake. V. Effect of cold-exposure during lactation in Mus musculus. *J. Exp. Biol.* **204**, 1967-1977.
Johnson, M. S., Thomson, S. C. and Speakman, J. R. (2001). Limits to sustained energy intake. I. Lactation in the laboratory mouse Mus musculus. *J. Exp. Biol.* **204**, 1925-1935.
Koteja, P., Swallow, J. G., Carter, P. A. and Garland, T., Jr (1999). Energy cost of wheel running in house mice: implications for coadaptation of locomotion and energy budgets. *Physiol. Biochem. Zool.* **72**, 238-249.
Koteja, P., Swallow, J. G., Carter, P. A. and Garland, T. (2001). Maximum cold-induced food consumption in mice selected for high locomotor activity: implications for the evolution of endotherm energy budgets. *J. Exp. Biol.* **204**, 1177-1190.
Koteja, P., Carter, P. A., Swallow, J. G. and Garland, T., Jr (2003). Food wasting by house mice: variation among individuals, families, and genetic lines. *Physiol. Behav.* **80**, 375-383.
Krol, E., Johnson, M. S. and Speakman, J. R. (2003). Limits to sustained energy intake VIII. Resting metabolic rate and organ morphology of laboratory mice lactating at thermoneutrality. *J. Exp. Biol.* **206**, 4283-4291.
Malisch, J. L., Saltzman, W., Gomes, F. R., Rezende, E. L., Jeske, D. R. and Garland, T., Jr (2007). Basal and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. *Physiol. Biochem. Zool.* **80**, 146-156.
Oklejewicz, M., Hut, R. A., Daan, S., Loudon, A. S. and Stirland, A. J. (1997). Metabolic rate changes proportionally to circadian frequency in tau mutant Syrian hamsters. *J. Biol. Rhythms* **12**, 413-422.
Perrigo, G. (1987). Breeding and feeding strategies in deer mice and house mice when females are challenged to work for their food. *Anim. Behav.* **35**, 1298-1316.
Perrigo, G. and Bronson, F. H. (1983). Foraging effort, food intake, fat deposition and puberty in female mice. *Biol. Reprod.* **29**, 455-463.
Perrigo, G. and Bronson, F. H. (1985). Behavioral and physiological responses of female house mice to foraging variation. *Physiol. Behav.* **34**, 437-440.
Rezende, E. L., Gomes, F. R., Malisch, J. L., Chappell, M. A. and Garland, T., Jr (2006a). Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. *J. Appl. Physiol.* **101**, 477-485.
Rezende, E. L., Kelly, S. A., Gomes, F. R., Chappell, M. A. and Garland, T., Jr (2006b). Effects of size, sex, and voluntary running speeds on costs of locomotion in lines of laboratory mice selectively bred for high wheel-running activity. *Physiol. Biochem. Zool.* **79**, 83-99.
Rhodes, J. S., Koteja, P., Swallow, J. G., Carter, P. A. and Garland, J. T. (2000). Body temperature of house mice artificially selected for high voluntary wheel-running behavior: repeatability and effect on genetic selection. *J. Therm. Biol.* **25**, 391-400.
Rhodes, J. S., Gammie, S. C. and Garland, T., Jr (2005). Neurobiology of mice selected for high voluntary wheel-running activity. *Integr. Comp. Biol.* **45**, 438-455.
Rikke, B. A., Yerg, J. E., Battaglia, M. E., Nagy, T. R., Allison, D. B. and Johnson, T. E. (2003). Strain variation in the response of body temperature to dietary restriction. *Mech. Ageing Dev.* **124**, 663-678.
Romijn, C. and Lokhorst, W. (1961). Some aspects of energy metabolism in birds. In *Proceedings Second Symposium on Energy Metabolism* (ed. E. Brouwer and A. J. H. van Es), pp. 49-58. Wageningen: EAAP.
Selman, C., Phillips, T., Staib, J. L., Duncan, J. S., Leeuwenburgh, C. and Speakman, J. R. (2005). Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition. *Mech. Ageing Dev.* **126**, 783-793.
Speakman, J. R. (1997). *Doubly Labelled Water: Theory and Practice*. London: Chapman & Hall.
Speakman, J. R. and Krol, E. (2005). Limits to sustained energy intake IX: a review of hypotheses. *J. Comp. Physiol. B* **175**, 375-394.
Speakman, J. R. and Selman, C. (2003). Physical activity and resting metabolic rate. *Proc. Nutr. Soc.* **62**, 621-634.
Swallow, J. G., Carter, P. A. and Garland, T., Jr (1998). Artificial selection for increased wheel running behavior in house mice. *Behav. Genet.* **28**, 227-237.
Vaanholt, L. M., Garland, T., Jr, Daan, S. and Visser, G. H. (2007). Wheel-running activity and energy metabolism in relation to ambient temperature in mice selected for high wheel-running activity. *J. Comp. Physiol. B* **177**, 109-118.
Visser, G. H. and Schekkerman, H. (1999). Validation of the doubly labeled water method in growing precocial birds: the importance of assumptions concerning evaporative water loss. *Physiol. Biochem. Zool.* **72**, 740-749.
Wiersma, P. and Verhulst, S. (2005). Effects of intake rate on energy expenditure, somatic repair and reproduction of zebra finches. *J. Exp. Biol.* **208**, 4091-4098.
Wiersma, P., Salomons, H. M. and Verhulst, S. (2005). Metabolic adjustments to increasing foraging costs of starlings in a closed economy. *J. Exp. Biol.* **208**, 4099-4108.